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DNA barcoding identifies a novel population of the imperiled Trinity Pigtoe, *Fusconaia chunii* (Lea, 1862) (Bivalvia, Unionidae), in the San Jacinto River drainage in Texas

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Abstract

The Trinity Pigtoe, *Fusconaia chunii* (Lea, 1862), is a freshwater mussel endemic to the Trinity River drainage in Texas. Here, we report the first population of *F. chunii* in the San Jacinto River drainage in Texas. We identified three specimens of *F. chunii* using DNA barcoding, which were morphologically indistinguishable from syntopic *Fusconaia flava* (Rafinesque, 1820). A similar issue occurs in the Trinity River drainage. *Fusconaia chunii* is listed as state threatened, and future research is necessary to assess its status within the San Jacinto River drainage.

Keywords

Conservation, freshwater mussel, North America, Pleurobemini

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Introduction

A critical aspect of biodiversity conservation is delineating the geographic ranges of species, which can be difficult in morphologically cryptic groups (Hey et al. 2003). This issue is exemplified in freshwater mussels (Bivalvia, Unionida, Unionidae), a diverse lineage of aquatic bivalves with 958 species recognized globally (Graf and Cummings 2021). Many lineages of freshwater mussels exhibit high levels of morphological variability, which has led to distributional information that may not accurately reflect species boundaries (Inoue et al. 2013; Froufe et al. 2016; Johnson et al. 2018; Bolotov et al. 2019; Smith et al. 2019). Issues with morphology-based taxonomy have highlighted distributional information as a major knowledge gap for many freshwater mussel species (Lopes-Lima et al. 2021), which is troubling considering 65% of species in North America are of conservation concern (Haag and Williams 2014).

Unionids in the North American tribe Pleurobemini Hannibel, 1912 are prone to high levels of misidentification due to subtle interspecific differences and high levels of intraspecific variation in shell morphology (Campbell and Lydeard 2012; Inoue et al. 2018). This has

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led to inaccurate taxonomic hypotheses for many species, and molecular data has shown to be useful in distinguishing species boundaries and their ranges in this group (Campbell and Lydeard 2012; Pieri et al. 2018; Morrison et al. 2021). The pleurobemine genus *Fusconaia* Simpson, 1900 consists of 13 species endemic to the United States and Canada (Williams et al. 2017; Pieri et al. 2018; Smith et al. 2021). Molecular research has led to numerous taxonomic changes in *Fusconaia* (Campbell and Lydeard 2012; Pfeiffer et al. 2016; Pieri et al. 2018; Smith et al. 2021), especially for species found in Texas: *F. askewi* (Marsh, 1896), *F. chunii* (Lea, 1862), *F. flava* (Rafinesque, 1820), *F. iheringi* (Wright, 1898), and *F. mitchelli* (Simpson in Dall, 1895). One example is the Trinity Pigtoe, *F. chunii*, which was recently elevated from synonymy and considered restricted to the Trinity River drainage in Texas (Pieri et al. 2018; Randklev et al. 2020). It was previously assumed to be a synonym of *F. askewi* or *F. flava* due to similarities in external shell morphology (Howells et al. 1996) (Fig. 1). However, a recent study by Pieri et al. (2018) demonstrated *F. chunii* (Trinity River drainage) was molecularly diagnosable from other *Fusconaia* species that occur in east Texas (*F. askewi* – Neches and Sabine River drainages; *F. flava* – Neches, Red, Sabine, San Jacinto, and Trinity River drainages) despite not being morphologically distinctive from co-occurring *F. flava* (Pieri et al. 2018). This finding has raised questions about the distribution of *F. chunii* in Texas and the need to DNA barcode *Fusconaia* specimens from other drainages, particularly those that historically shared some level of connectivity to the Trinity River basin.

Fusconaia chunii is listed as state threatened and considered to be a species of greatest conservation need by the state of Texas (TPWD 2020). Thus, confirming the presence of *F. chunii* outside the Trinity River drainage will have major conservation implications—an extension of its geographic range will increase its overall resiliency to stochastic events and anthropogenic impacts. In this study, we report the first population of *F. chunii* in the San Jacinto River drainage, which provides direction for freshwater mussel conservation in Texas.

Methods

Taxon sampling and molecular data generation. We compiled (n = 122) or generated (n = 29) molecular data for 151 individuals of *F. askewi* (n = 57), *F. chunii* (n = 43), and *F. flava* (n = 51) collected from five river drainages in Texas: Neches, Red, Sabine, San Jacinto, and Trinity (Fig. 2; Table 1; Supplementary Table S1). Novel molecular data was generated from specimens collected between 2017 and 2021 in the Neches, Red, Sabine, and San Jacinto River drainages. Initial identifications were based on drainage of capture and external shell morphology. All individuals collected from the San Jacinto River drainage were tentatively identified as *F. flava*. Specimens were deposited in the Joseph Britton Freshwater Mussel Collection and are currently housed at the Texas A&M AgriLife Center at Dallas.

Considering phylogenetic relationships in *Fusconaia* are well characterized (Pieri et al. 2018; Smith et al. 2021), we did not include other *Fusconaia* spp. in downstream analyses. For novel sequence data, DNA was extracted from fresh mantle tissue using the Qiagen PureGene DNA extraction kit with the standard extraction protocol (Hilden, Germany). We amplified and sequenced a 658 base pair segment of the mitochondrial protein coding gene *COX1* using primers presented by Campbell et al. (2005): F: 5′-GTTCCACAAATCATAAGGATATTGG-3′ and R: 5′-TACACCTCAGGGTGACCAAAAAACCA-3′. PCR reactions were performed using a 12.5 µl mixture of distilled deionized water (4.25 µl), MyTaqTM Red Mix (6.25 µl) (Bioline), primers (0.5 µl each at 10 µM), and DNA template (1 µl at 50 ng/µl). Thermal cycling conditions followed Johnson et al. (2018). PCR products were sent to the DNA Sequencing Facility at the University of Texas at Austin (Austin, Texas, USA) for bidirectional sequencing on an ABI3730. Geneious Prime v. 2021.2.2 (https://www.geneious.com) was used to assemble and edit consensus sequences, and loci were independently aligned using MAFFT v. 7.311 (Katoh and Standley 2013). Consensus sequences were translated into amino acids to ensure absence of stop codons and gaps.

Molecular analyses. Phylogenetic inference was performed in BEAST v. 2.6.6 (Bouckaert et al. 2019). We included a *COX1* sequence for *Pleurobema clava* (Lamarck, 1819) to serve as an outgroup for the analysis based off the findings from previous phylogenetic studies (Campbell and Lydeard 2012; Inoue et al. 2018). Specifically, these studies consistently resolved species in *Pleurobema* as sister to *Fusconaia*. Given *P. clava* is the type species of *Pleurobema*, we considered it an appropriate outgroup. Before the analysis, the best nucleotide substitution model was determined using ModelFinder (Kalyaanamoorthy et al. 2017). We used a strict molecular clock, and Yule process was used as the tree prior. The BEAST analysis was run for 1.5×10^7 generations, sampling every 5000 generations with an initial 10% burn-in. Effective sample size greater than 200 for each parameter was ensured using Tracer v. 1.7 (Rambaut et al. 2018), and a maximum clade credibility tree was created using TreeAnnotator v. 2.6 (Bouckaert et al. 2019).

To further explore genetic variation between and within *F. askewi*, *F. chunii*, and *F. flava*, we constructed a TCS haplotype network from the *COX1* alignment using PopART (Clement et al. 2000; Leigh and Bryant 2015). Individuals were grouped by species and drainage of capture (Neches, Red, Sabine, San Jacinto, and Trinity). Missing data was handled using complete deletion. We visually compared sequences in MEGA11 (Tamura et al. 2021) to calculate the number of sites in our alignment that diagnosed *F. chunii* from *F. flava*.

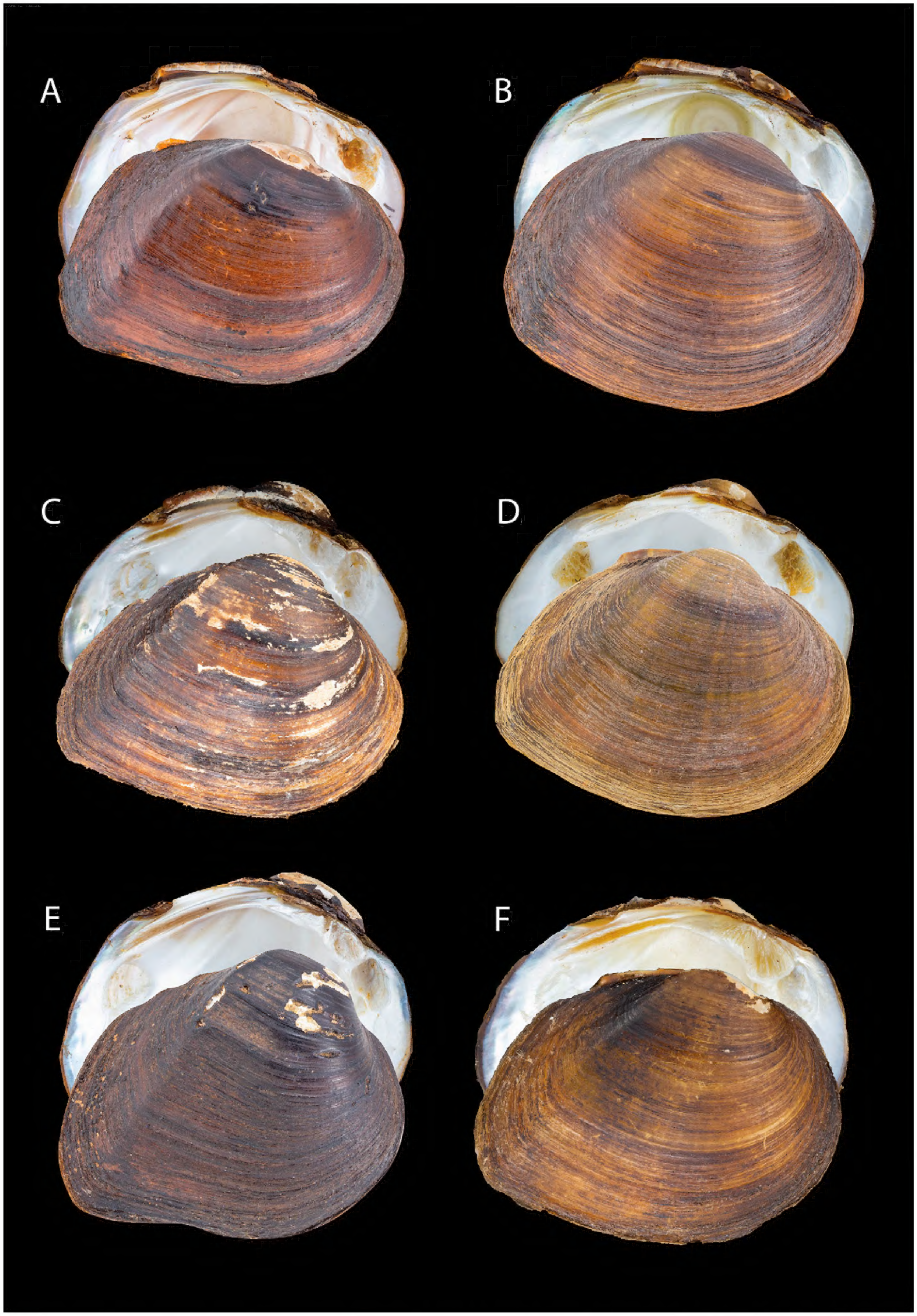

Figure 1. Representative specimens of *Fusconaia askewi*, *F. chunii*, and *F. flava*. **A.** *F. askewi* from the Neches River drainage (JBFMC 8096.1; 59 mm). **B.** *F. askewi* from the Neches River drainage (JBFMC 8096.3; 49 mm). **C.** *F. chunii* from the Trinity River drainage (JBFMC 8035.1, 74 mm). **D.** *F. chunii* from the Trinity River drainage (JBFMC 8284.4; 56 mm). **E.** *F. flava* from the Red River drainage (JBFMC 8249.1, 55 mm). **F.** *F. flava* from the San Jacinto River (JBFMC 8031.3, 41 mm).

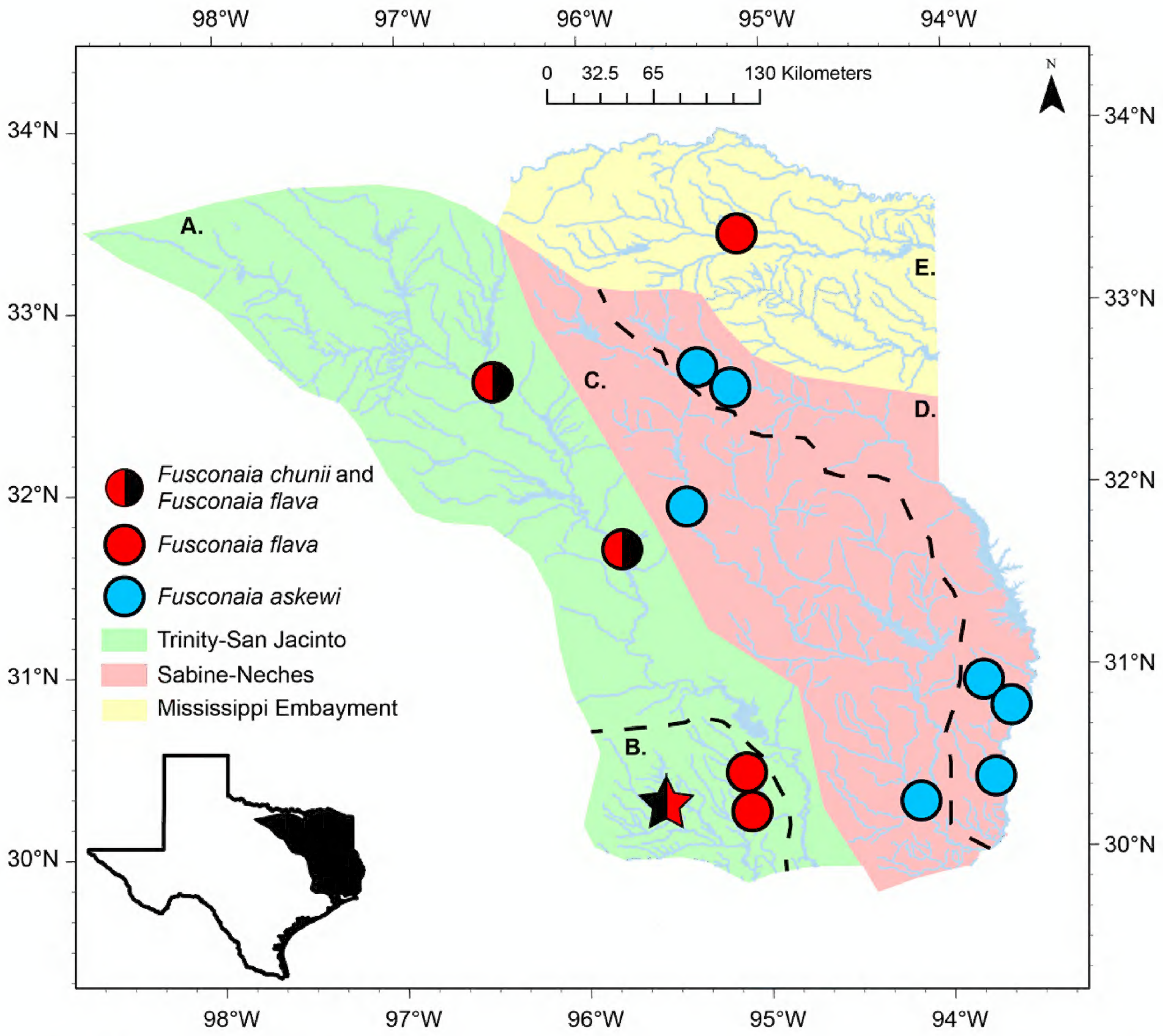

Figure 2. Collection localities for *Fusconaia* specimens used in this study. Circles represent unique collection localities and coloration corresponds to species detected at the locality. The star corresponds to the collection locality for the three new records of *F. chunii* in the San Jacinto River drainage (JBFMC 11063). Limits of biogeographic provinces are colored based on designations by de Moulpied et al. (2022). River drainages are as follows: **A** = Trinity, **B** = San Jacinto, **C** = Neches, **D** = Sabine, and **E** = Red. In cases when two river drainages are present in a province (e.g., San Jacinto and Trinity), drainages are separated by a dotted black line.

Results

Family Unionidae Rafinesque, 1820
Subfamily Ambleminae Rafinesque, 1820
Tribe Pleurobemini Hannibal, 1912
Genus *Fusconaia* Simpson, 1900

***Fusconaia chunii* (Lea, 1862)**

New records. United States of America – **Texas** • San Jacinto River > West Fork San Jacinto River > Lake Creek; 30°15′12″N, 095°34′45″W; 11.VIII.2021; Chase Smith and Charles Randklev leg.; GenBank OM471685–OM471687; Joseph Britton Freshwater Mussel Collection

Table 1. Material examined in this study for molecular analyses with collection locality, number of individuals examined, and Joseph Britton Freshwater Mollusk Collection catalog numbers.

Taxa (sample size)	Drainage	Catalog numbers (sample size)
Fusconaia askewi (24)	Neches	JBFMC8096 (7); JBFMC8309 (16); JBFMC9509 (1)
Fusconaia askewi (33)	Sabine	JBFMC8110 (4); JBFMC8116 (1); JBFMC8192 (1); JBFMC8252 (3); JBFMC8295 (20); JBFMC10009 (4)
Fusconaia chunii (40)	Trinity	JBFMC8035 (8); JBFMC8077 (3); JBFMC8284 (16); JBFMC8290 (13)
Fusconaia chunii (3)	San Jacinto	JBFMC11063 (3)
Fusconaia flava (2)	Red	JBFMC9588 (2)
Fusconaia flava (24)	Trinity	JBFMC8035 (2); JBFMC8063 (2); JBFMC8077 (1); JBFMC8284 (13); JBFMC8290 (6)
Fusconaia flava (25)	San Jacinto	JBFMC8031 (4); JBFMC8463 (5); JBFMC9502 (9); JBFMC11054 (1); JBFMC11062 (6)

11063, 3 wet specimens, 95% EtOH.

Identification. Shells 33–35 mm in length. Shells moderately thick, moderately compressed to inflated; outline subtriangular to subrhomboid; posterior ridge high and narrowly rounded, ends at a blunt point; sulcus present anterior to the posterior ridge; posterior slope slightly concave. Shell color yellow to reddish-brown; green or brown rays; surface dull to subglossy. Shell texture smooth. Umbo low and narrow, unsculptured; umbo cavity moderately shallow. Pseudocardinal teeth large and somewhat compressed, rough, and triangular; 2 pseudocardinal teeth in left valve. Anterior tooth compressed, parallel to the hinge line; 1 in right valve. Lateral teeth short to moderately long, thick, and slightly curved; 2 in left valve, 1 in right valve. Interdentum moderately long, narrow to wide. Nacre white with salmon or rose highlights; iridescent posteriorly. Soft tissues white to light brown in most individuals, but foot may be a brighter color, primarily orange, and may be red internally. Gills white, but may be pink or red when gravid.

Specimens of *F. chunii* were diagnosable from other freshwater mussel species using molecular or morphological characters. *Fusconaia chunii* is easily diagnosable from most sympatric species due to its subtriangular to subrhomboid shell outline and well-developed posterior ridge. Some specimens of *F. chunii* may resemble *Cyclonaias pustulosa* (Lea, 1831) or *Pleurobema riddellii* (Lea, 1861). We differentiated *F. chunii* from these two species by the lack of pustules, reduced umbo, and orange to light brown soft parts (e.g., foot, gills, mantle). Specimens of *F. chunii* are morphologically indistinguishable from *F. flava* (Fig. 3) and were identified using *COX1* sequence data. Our phylogenetic analysis resolved three specimens tentatively identified as *F. flava* from the San Jacinto River drainage monophyletic with *F. chunii* from the Trinity River drainage (Fig. 4). The TCS haplotype network showed these specimens shared haplotypes with known *F. chunii* from the Trinity River (Fig. 4), and sequences were diagnosable from *F. flava* at 19 sites.

Discussion

The distribution of freshwater mussels in Texas generally follows provinces defined by de Moulpied et al. (2022). In the case of *Fusconaia* in east Texas, species clearly follow province designations, with *F. askewi* restricted to the Sabine-Neches province, *F. chunii* in the Trinity-San Jacinto province, and *F. flava* throughout the Sabine-Neches and Trinity-San Jacinto provinces (Fig. 2). Pieri et al. (2018), who elevated *F. chunii* from synonymy, restricted the distribution of *F. chunii* to the Trinity River drainage considering all examined individuals from the San Jacinto River drainage were molecularly identified as *F. flava*, albeit based on limited sampling ($n = 4$). This was an intriguing conclusion considering nearly all taxa found in the Trinity River are assumed to occur in the San Jacinto River drainage currently or historically (Howells et al. 1996). Here, we report an extant population of *F. chunii* in the San Jacinto River drainage, which increases the known range of the species. We find it unlikely that this population was introduced given our findings align

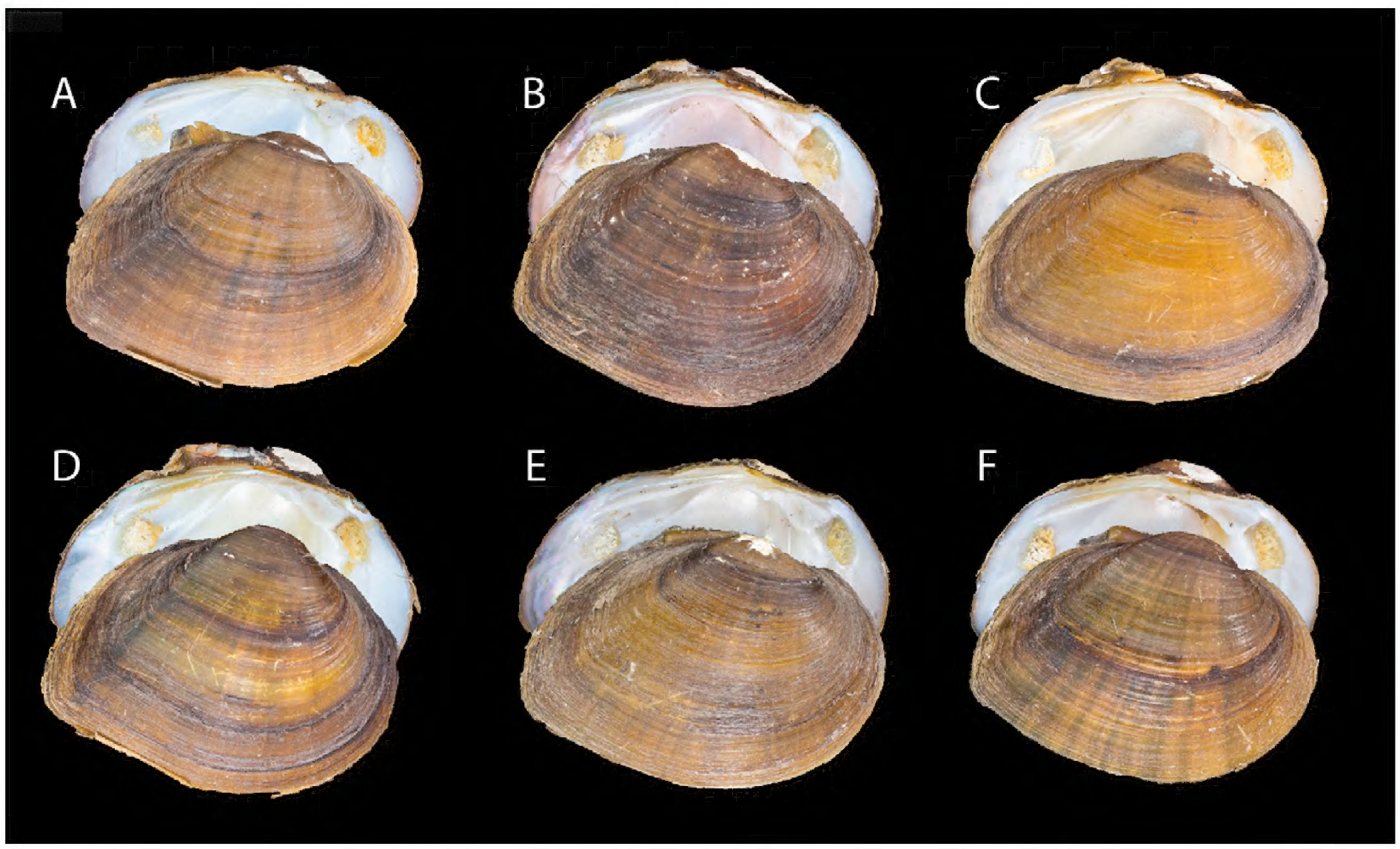

Figure 3. Syntopic *Fusconaia chunii* (**A–C**) and *F. flava* (**D–F**) specimens from the San Jacinto River drainage (Lake Creek). **A.** JBFMC 11063.1 (35 mm). **B.** JBFMC 11063.2 (33 mm). **C.** JBFMC 11063.3 (35 mm). **D.** JBFMC 11062.1 (49 mm). **E.** JBFMC 11062.3 (33 mm). **F.** JBFMC 11062.4 (30 mm).

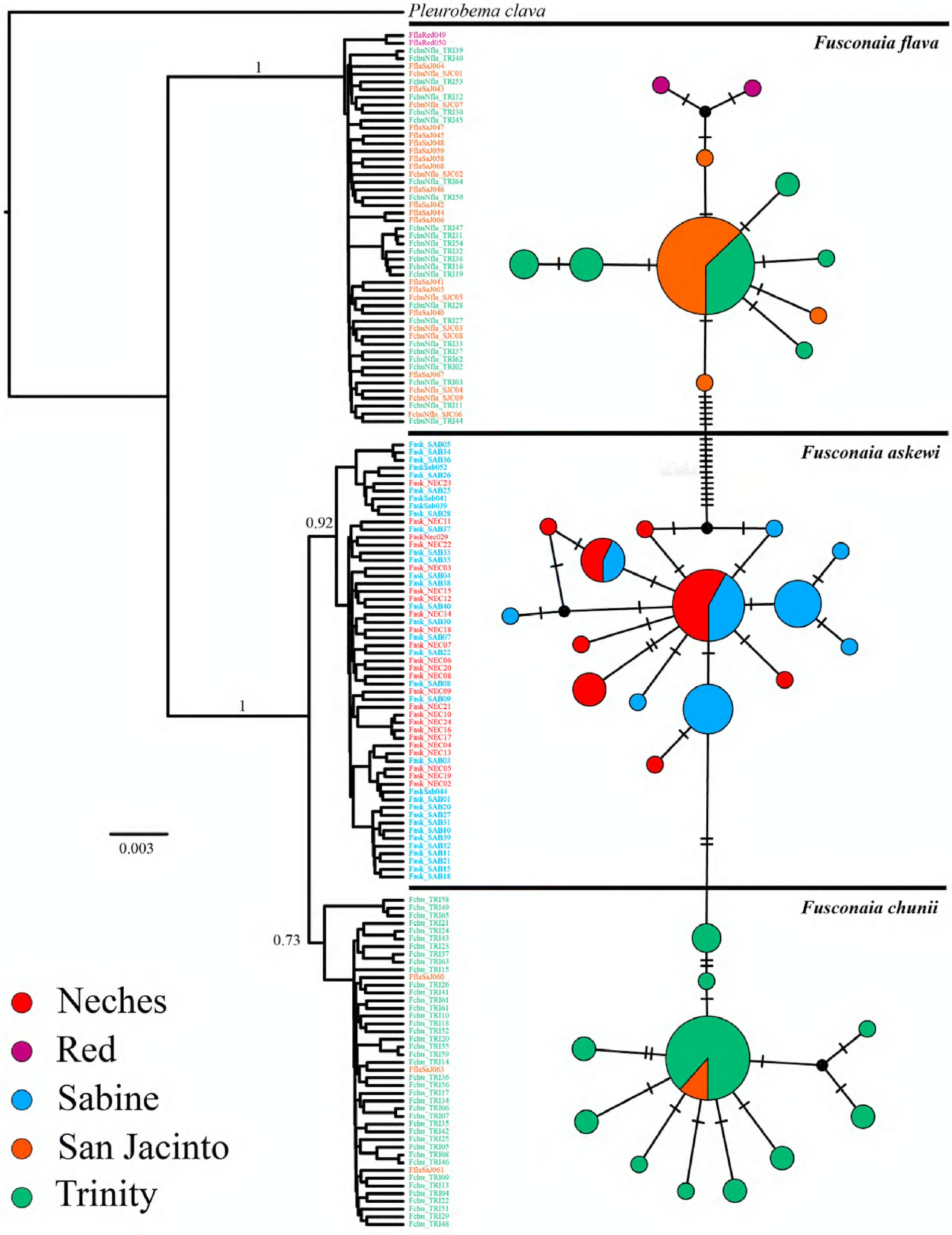

Figure 4. Maximum clade credibility tree generated by BEAST and haplotype network of *Fusconaia askewi*, *F. chunii*, and *F. flava* based on *COX1*. Numbers above or beside branches in the phylogram represent posterior probability support. Tip labels and circle colors correspond to drainage of capture. In the haplotype network, each circle represents a unique haplotype and size is relative to the number of individuals. Black circles represent hypothetical unsampled haplotypes. Hash marks represent nucleotide substitutions.

with both biogeographic patterns and previous researchers that have hypothesized specimens similar to *F. chunii* may have been present in the San Jacinto River drainage historically (Pilsbry 1891; Howells et al. 1996).

DNA barcoding was able to identify *F. chunii*, however, specimens were indistinguishable from *F. flava* using morphological characters (Fig. 3), which has also been observed in the Trinity River drainage (Pieri et al. 2018). For the San Jacinto specimens, we were unable to find diagnosable morphological shell characters, internal or external, or soft part anatomy for distinguishing *F. chunii* from *F. flava*. We acknowledge that sample sizes from the San Jacinto were low in this study (*F. chunii*: $n = 3$; *F. flava*: $n = 25$) and more robust investigations are needed to evaluate whether diagnosable features exist. We suspect this is probably not the case given previous studies have also failed to find shell characters to distinguish between sympatric *F. chunii* and *F. flava* in the Trinity River drainage (Pieri et al. 2018). Because of this, characterization of life history traits, such as brooding phenology, may be more promising since these traits have been shown to differentiate freshwater mussel lineages with distinct evolutionary trajectories (Sietman et al. 2018). We hypothesize *F. askewi* and *F. chunii* likely display similar life history characteristics that may distinguish both taxa from sympatric *F. flava* given their limited molecular divergence (Fig. 4). Future life-history studies characterizing brooding phenology, host attraction strategy, and host use may shed light on why *F. askewi* and *F. chunii* remain reproductively isolated from *F. flava*.

Fusconaia chunii is listed as threatened by the state of Texas, and previous studies have suggested the distribution of *F. chunii* appears to be significantly reduced in the Trinity River (Burlakova et al. 2012; Pieri et al. 2018). Recent survey data suggests the species has extant populations occurring throughout the Trinity River drainage upstream of Lake Livingston and a single record from Menard Creek, a tributary of the lower Trinity River (Randklev et al. 2020). However, future research is necessary to assess its status in the San Jacinto River drainage. We were only able to confirm an extant population of *F. chunii* in Lake Creek, a tributary of the West Fork San Jacinto River, and the species is likely more widespread in the West Fork San Jacinto River between lakes Houston and Conroe. This is supported by historical collections from the West Fork San Jacinto River downstream of the confluence with Lake Creek that have been identified as *F. chunii* (Mississippi Museum of Natural Science 11910; Florida Museum 565542), albeit we cannot confirm these records without molecular characters. We were also unable to identify *F. chunii* in the East Fork of the San Jacinto River despite generating molecular data from 25 individuals putatively identified as *F. flava*. However, it is plausible that *F. chunii* is sympatric with *F. flava* in the East Fork San Jacinto River, as has been hypothesized by previous collectors (Illinois Natural History Survey 7654; Mississippi Museum of Natural Science 11915). Further molecular sampling in the waterbody and its tributaries may confirm its presence.

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Authors' Contributions

Conceptualization: CHS. Data curation: CHS. Formal analysis: CHS. Funding acquisition: CR Randklev. Investigation: CH Smith. Methodology: CHS, CR Robertson, CR Randklev. Resources: CR Randklev. Visualization: CR Robertson. Writing – original draft: CHS. Writing – review and editing: CR Robertson, R Randklev.

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Supplemental Data

Table S1. Metadata associated with material examined in this study, including identifiers used in the phylogenetic analysis, collection locality (i.e., drainage, waterbody, latitude, and longitude), Joseph Britton Freshwater Mollusk Collection catalog number, *COX1* GenBank accession number, and the source study of the *COX1* sequence used for each specimen.